

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/AU86/00139 <b>(22) International Filing Date:</b> 15 May 1986 (15.05.86)  <b>(31) Priority Application Number:</b> PH 0571 <b>(32) Priority Date:</b> 15 May 1985 (15.05.85) <b>(33) Priority Country:</b> AU  <b>(71) Applicant (for all designated States except US):</b> COMMONWEALTH SERUM LABORATORIES COMMISSION [AU/AU]; 45 Poplar Road, Parkville, VIC 3052 (AU).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> DAVIES, Jeffrey, R. [AU/AU]; 88 Hunter Street, Brunswick West, VIC 3055 (AU). HEARN, Milton, T., W. [AU/AU]; 263 Union Road, Balwyn, VIC 3103 (AU). MARLEY, Patrick, B. [AU/AU]; 71 Holmes Road, Moonee Ponds, VIC 3039 (AU).		<b>(74) Agent:</b> SLATTERY, John, Michael; Davies & Collison, 1 Little Collins Street, Melbourne, VIC 3000 (AU).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent), US.  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PREPARATION OF MONOMERIC IgG  <b>(57) Abstract</b>  <p>A method for the purification of an IgG containing material, comprises the steps of: (i) fractionating an immunoglobulin G-containing material on a microparticulate, strong anion exchange resin having meso- and macro- porous surfaces, and (ii) recovering a purified IgG fraction from the resin by elution. The method may further comprise removal of Kallikrein-like esterase and other protease enzyme or protease zymogen activity from an IgG-containing material which comprises the steps of: (i) contacting the IgG-containing material with a protein-binding dye immobilised on a macroporous, mechanically stable gel support, and (ii) recovering the enzyme and zymogen-depleted IgG-containing material. The IgG-containing material may for example be Cohn Fraction II paste or powder.</p>		

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PREPARATION OF MONOMERIC IgG1. Field of the Invention

This invention relates to a new method for the processing of normal or hyperimmune human gammaglobulin (IgG), for example the product obtained from Cohn Fraction II or other suitable materials, whereby aggregated and monomeric IgG are resolved to produce a product consisting essentially of monomeric IgG which can be given by intravenous injection. The invention further relates to various methods for the removal of contaminant proteins including IgA, IgM, plasminogen, plasmin, Factor XII, prekallikrein activator (PKA), kallikrein and other kallikrein-like esterase activities.

It is an object of the present invention to go some way toward overcoming certain disadvantages associated with currently available IgG preparations. It has been found that the method of the present invention allows removal of aggregated and dimeric forms from monomeric forms of immunoglobulin, all of which are usually present in IgG-rich fractions such as Cohn Fraction II, and consequently reduces so-called anticomplementary activity (ACA), and further, allows significant removal of other contaminant proteins,

notably those which can lead to the generation of kinins.

## 2. Background to the Invention

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Human blood serum or plasma is rich in immunoglobulin G (IgG) directed against antigens from various sources with a range of different properties. For example, invasion by viral or bacterial  
10 micro-organisms and the resultant exposure of the individuals to specific immunogens commonly results in the production of specific antibodies of the IgG class by B cell lymphocytes. These antibodies effectively control the further proliferation of the micro-organism,  
15 in conjunction with macrophages and plasma proteins of the complement system, and the process is known as humoral immunity.

The ability of an individual to mount a  
20 humoral response against a specific immunogen varies in degree and with the nature of the immunogen. Humoral immunity is at its weakest between the 6th and 24th months of life and continues to develop over the first twenty years of life. Some humans have a poor ability  
25 to produce IgG and hence have a compromised immunity. These conditions can be hereditary e.g. infantile X-chromosome linked hypogammaglobulinaemia (Bruton's disease) or be acquired, usually presenting as a selective or partial antibody deficiency syndrome. Such  
30 persons normally suffer from different, frequently recurring infections and whenever possible are supplied with IgG prepared from a pool of normal donors' blood. Passive immunization with IgG has also become an  
35 important option in the treatment and prevention of

infectious diseases, especially in cases of infection with bacteria resistant to antibiotic treatment and more recently in the treatment of idiopathic thrombocytopenic purpura. Hence, the need for a preparation of human IgG is well established and proven.

Since the clinical introduction of IgG preparations (i.e. gammaglobulin concentrates) by Stokes et al. in 1944 (Stokes, J., Maris, E.P. and Gellis, S.S., J.Clin.Invest. (1944) 23 531) many attempts have been made to produce a preparation which could be given intravenously without causing side effects. Nevertheless, because of the difficulties referred to below, the regimen of intra-muscular injection of the Cohn II fractionated IgG, used by Bruton in 1952 (Bruton, O.C. Pediatrics (1952) 9 72-727) in the treatment of agammaglobulinaemia, became standard practice for many years and in fact has only recently been outdated in the USA. The intramuscular route has a number of important disadvantages compared with the intravenous route. For example, the injection volume is limited and is especially problematical in children with a small muscle mass, absorption from the injection site is relatively slow, a major proportion of the immunoglobulin is broken down by proteolysis at the site of the injection, consequently plasma levels are invariable and unpredictable, and the injection is painful.

From the disadvantages outlined above, it is clear that the intravenous route of injection of IgG is the route of choice. An important extra advantage is that more effective use of IgG can be made. For example, the dose of anti D IgG required to neutralise D

Rhesus positive cells acquired from the foetus during pregnancy is several fold less when given intravenously than when given intramuscularly. However, as already mentioned, the earlier preparations of IgG could not be  
 5 given intravenously because of the side effects they caused. (Janeway, C.A. et al., New Engl.J.Med. (1968) 278 919). Most of the preparations used presently are still not without unwanted effects and consequently are usually administered very slowly in a diluted form,  
 10 especially in patients with antibody deficiency syndromes who are more sensitive to the adverse properties of IgG preparations than their normal counterparts.

15 Adverse reactions to the early gammaglobulin concentrates were severe and resulted in a shock reaction often caused by hypotensive circulatory failure. Many of the second generation IgG concentrates i.e. those modified for intravenous injection, still  
 20 cause reactions such as nausea, vomiting, pyrexia, rigors, backache, chest constriction, flushing and hypotension, especially in susceptible patients such as those suffering from agammaglobulinaemia. The anaphylactoid and anaphylactic reactions have been  
 25 attributed to the anticomplementary activity (ACA) of aggregated IgG, found in variable amounts in IgG preparations, as aggregated IgG was shown to inactivate complement i.e. to activate the complement cascade, in a similar manner to antigen-antibody complexes (Ishizaka,  
 30 T., Ishizaka, K. & Boros, T., J.Immunol. (1961) 87 433).

Activation of the complement cascade generates, inter alia, anaphylatoxins which have dilator effects on blood vessels and which increase the  
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permeability of capillaries. An uncontrolled activation could therefore lead to hypotension and circulatory collapse.

5           The complement fixing sites have been shown to  
be localised in the Fc fragment of the IgG molecule  
(Taranta, A. & Franklin, E.C., Science (1961) 134 1981)  
and this has led subsequently to products where the Fc  
portion of IgG is removed enzymatically (see below). It  
10 has also been shown that two Fc fragments must be  
brought into apposition to fix complement (Isliker, H.,  
Jacot-Guillarmod, H. & Jalon, J.C., Ergebn.Physiol.  
(1965) 56 67). This "non-specific activation" of the Fc  
region appears to be the result of damage to the IgG  
15 during extraction but the mechanism is not yet  
understood. IgG may undergo a conformational change  
leading to exposure of previously cryptic sites which  
are then able to bind to and activate Fc receptor sites  
on cells and tissues. It follows that methods for the  
20 preparation of the IgG must avoid processes likely to  
activate the IgG and emphasizes the desirability of  
having IgG in the monomeric form.

          Clinical proof that aggregates cause or are  
25 partially responsible for adverse reactions is still  
lacking. This unsatisfactory situation stems from the  
lack of suitable pharmacological models for detecting  
and quantitating factors responsible for adverse  
reactions. At present it is difficult to investigate,  
30 under standardized clinical conditions, all of the  
factors that might underlie adverse reactions because  
the mechanisms of the adverse effects are poorly  
understood and the current intravenous IgG preparations  
are so heterogeneous.

The abilities of manufacturers to make IgG preparations safe for intravenous administration have been hampered by this incomplete knowledge of the mechanisms of the adverse reactions. The heterogeneity of the various manufacturers' products has also contributed to the controversy in this area. Not surprisingly therefore, there exists in the patent and scientific literature a multitude of methods for the purification of IgG. Some of the more well known methods are discussed briefly below.

- (a) Fractionation of IgG: Several methods are known for the fractionation of IgG from starting materials of human or animal origin such as blood plasma, blood serum, placentae and other fluids. For example, fractionation with alcohols at a low temperature, (Cohn, E.J. et al., J.Am.Chem.Soc. (1946) 68 459-475; Oncley, J.L., Melin, M., Richert, D.A., Cameron, J.W. and Gross, Jr., P.M., J.Am.Chem.Soc. (1949) 71 541; Kistler, P. and Nitschmann, H., Vox Sang. (1962) 7 414-424); fractionation with Rivanol (a trade mark for acrinol) - ammonium sulphate, (Horejisi, J. and Smetana, R., Acta Medica Scandinavica (1956) 155 65-70) and ion-exchange chromatography (Hope, H. et al., Munchen Medizinische Wochenschrift (1967) 34 1749-1752).

Furthermore, Canadian Patent 1 137 413 discloses that by modification of these methods by carrying out the fractionations in the presence of at least one water soluble basic nitrogen-containing organic compound having a dissociation constant of 7 or



less, or an acid salt of the same, a product with higher monomer content can be obtained.

5 (b) Removal of IgG Aggregates: Ultracentrifugation can  
produce a product with low ACA which is well  
tolerated intravenously (Barundan, S. et al., Vox  
Sang. (1962) 7 157-174), but preparative  
ultracentrifugation is an impractical proposition.  
The removal of the IgG aggregates has also been  
10 attempted through adsorption by means of activated  
charcoal (Steinbuch, M., Vox Sang. (1967) 13 103),  
with starch, with silicates (German  
Offenlegungsschrift 26 58 334) as well as through  
precipitation with polyethylene glycols (German  
15 Offenlegungsschrift 27 51 717) (cf. Polson, A.  
et al. Vox Sang. (1972) 23 107-118; Schneider, W.  
et al. Vox Sang. (1976) 31 141-151). None of  
these methods permits complete removal of the  
anticomplementary activity.

20 (c) Enzymatic Degradation: Enzymatic splitting of IgG  
with pepsin (Schultze, H.E. and Schwick, G.,  
Dtsch. Med.Wschr. (1967) 87 1643), has been  
employed to produce a commercial preparation devoid  
25 of ACA. For example, French patent 2,382,000  
describes such a product. This method effectively  
decreases ACA without reducing antibody titres.  
However, the Fc fragment is completely destroyed,  
thus removing the tissue binding capacity of the  
30 IgG molecules. This feature, and the loss of small  
fragments in the urine (Barandun et al. supra),  
account for the greatly reduced half-life of this  
preparation in the body (Koblet, H., Diggelmann,  
H., Barandun, S. and Gerber, H.

Bibl.Haemat.(Basel) (1965) 23 1102). Furthermore, with the Fc fragment removed such a preparation is unable to pass the placental barrier.

5 Treatment of IgG with human plasmin can result in its cleavage into three components of 50,000 molecular weight and a product devoid of ACA (Sgouris, J.T., Vox Sang. (1967) 13 71). When sufficiently low levels of plasmin are used, only 10 15% of the molecules are cleaved, with 85% remaining as intact gamma globulin. (Sgouris, supra.) The intact gamma globulin remaining undigested shows little anticomplementary activity and has been administered intravenously without 15 adverse reactions. (Hinman, J. et al., Vox Sang. (1967) 13 85.) The material thus prepared appears to retain in vitro and in vivo protective activity. (Fitzpatrick, F.K., Vox Sang. (1967) 13 85). One disadvantage of this approach is that the plasmin 20 cannot be completely removed. Thus, degradation continues even when the material is stored at 4°C. A plasmin-treated IgG preparation is described in the German Offenlegungsschrift 27 52 694.

25 Barandun, S. et al (supra) showed that incubation of gamma globulin at pH 4.0 at 37°C reduces the anticomplementary activity; for example incubation for 24 hours completely eliminated it. It has been suggested that this result may arise from the 30 activity of a small quantity of serum enzyme present as an impurity in the gamma globulin. (Blatrix, C., et al., Presse Med. (1969) 77 635-637). As with the plasmin treated gamma globulin, this "pH 4.0 gamma globulin" has been 35

found to regain anticomplementary activity before administration to a patient. (Malgras, J. et al., Rev.Franc.Trans. (1970) 13 173).

5 Both plasmin treated gamma globulin and pH 4.0 gamma globulin have shorter half-lives in vivo than unmodified gamma globulin, e.g. 14-16 days compared with 20 days for unmodified IgG (Koblet, H. et al., Vox Sang. (1967) 13 93; Merler, E. 10 et al Vox Sang. (1967) 13 103).

(d) Chemical Modification: A number of methods are available from the literature which have found commercial application, as shown in the following 15 examples:-

i) Blocking of the complement receptors of the Fc segment of the IgG with  $\beta$ -propiolactone (Stephan, W., Vox Sang. (1975) 28 422-437). The products so 20 obtained no longer fix any complement and consist of monomers to the extent of 90%. However, the biological half-life is reduced to 4 to 12 days (European Patent Application 13,901; Barandun, S. et al, Monograph. Allergy, (1975) 9 39-60 Karger, 25 Basel).

ii) Reduction and sulphonation of the disulphide bridges of the IgG molecule greatly reduces the anticomplementary activity (Yamanaka, T. et al. Vox Sang. (1979) 37 14-20; further described in 30 Canadian Patent 1 128 418 and U.S. Patent 4,168,303).

iii) Partial reduction and alkylation (Schroder, D.D. et al., Vox Sang. (1981) 40 383-394 and further described in U.S. Patent No. 3,903,262) or 35

amidation (German Offenlegungsschrift 24 42 655) produces a less reactive preparation.

However, changes in the molecular structure, loss of some important physiological functions and the appearance of new antigenic determinants in the IgG molecule cannot be excluded by these chemical interventions.

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- 10 (d) Ion Exchange Chromatographic methods: Preparative methods for immunoglobulin G (IgG) from human plasma and serum based on ion exchange chromatography are well established (Baumstark, et al, Archiv.Biochem. & Biophys. (1964) 108
- 15 514-522; Webb, A.J., Vox Sang. (1972) 23 279-290). The use of DEAE cellulose to remove unwanted components such as IgG aggregates and other undesirable proteins from Cohn Fraction II to yield a product with low ACA has been described (Habeeb, A.F.S.A., et al, Vox Sang. (1977) 32 143-158; U.S. Patent 4,312,949) as has the use of DEAE Sephadex
- 20 A-50 (Patent Application PCT/US83/01016) which is claimed to be useful for the removal of prothrombin-complex proteins. Purification of hyperimmune IgG such as Rho (D) and antitetanus immunoglobulin by ion exchange (DEAE Sephadex) has also been described (Hoppe, et al., Vox Sang. (1973) 25 308-316; Friesan, et al, J.Appl.Biochem. (1981) 3 164-175) and ion exchange chromatography
- 25 has also been used in the purification of sulphonated monomeric IgG (Canadian Patent 1,128,418).
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Cation exchangers such as carboxymethylcellulose have been described for the partial resolution of IgG aggregates from monomers (Australian Patent Application No. AU A91328/82).

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Combined ion exchange (DEAE Sepharose Fast Flow) and affinity chromatography (Arginine-Sepharose 4B and Benzamidine-Sepharose 6B) have been reported to remove aggregates, fragments and PKA from IgG concentrates, at the low mg level (Berglof, J.H. and Eriksson, S. 18th Cong.Int.Soc.Blood Transfusion Munich 1984).

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One potential and especially desirable feature of ion exchange chromatography is its ability to remove or reduce the levels of contaminating hepatitis B surface antigen as for example when IgG is bound onto and preferentially eluted from DEAE Sephadex or QAE Sephadex, as described in Australian Patent Application No. Au-A-17277/83.

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There are also some well known disadvantages of traditional ion exchange chromatography. For example, methods which depend on monomeric IgG binding to the ion exchange material, and then being preferentially eluted, suffer from the limited binding capacity of the material and the general slowness of the process. Often ion exchange chromatography is combined with an  $\text{SiO}_2$  absorption step to absorb lipid substances and pro-enzymes but this contributes to significant losses of IgG ( 20%). Another disadvantage is that chromatography itself can bring about IgG aggregation. For example, Patent Application

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PCT/US83/01016 deals with the inclusion of various substantially non-surface active stabilizers to the IgG to overcome this tendency when IgG is purified on anion exchange resins.

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Flow rates on traditional ion exchange media have typically not exceeded 25-30 cm/h of linear flow velocity although agarose gels with improved cross-linking have been used at flow rates of 120 cm/h. Further, these conventional ion exchange resins can show other disadvantages which limit their usefulness for the rapid high resolution separation of biopolymers, including immunoglobulin preparations, such as,

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1. poor mechanical stability,

2. resin deformation due to the choice of eluent conditions, flow rate, temperature etc.,

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3. polydispersity of the resin particles in terms of both particle diameter distributions and pore size distributions,

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4. low loading capacities under dynamic conditions,

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5. difficulties in regeneration of the resin following usage due to mechanical, physical or chemical changes in the characteristics of the particle,

6. low resolution per unit time at high linear flow velocities,

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7. problems associated with the limitation of the choice of eluent composition required to achieve optimal resolution at a particular throughput.

5 8. significant losses of biological activity due to the large residence and separation times which may be required due to the low rate of zone development, i.e. the lower linear flow velocities which are possible.

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As previously outlined, it is an object of the present invention to avoid the disadvantages associated with these known methods, and products, and to provide a method for the resolution of immunoglobulin G-containing fractions so as to provide a product consisting essentially of monomeric IgG.

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According to a first aspect of the present invention, there is provided a method for the purification of an IgG-containing material which comprises the steps of

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(i) fractionating an immunoglobulin G-containing material on a microparticulate, strong anion exchange resin having meso- and macro-porous surfaces, and (ii) recovering a purified IgG fraction from said resin by elution.

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In this aspect, the invention also extends to the purified IgG obtained by the above described method.

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The microparticulate anion exchange resin may, for example, have nominal particle diameters of 3, 5, 10, 30 and 90  $\mu\text{m}$ , with narrow particle diameter distribution and narrow pore size distribution, based on

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porosity of  $> 10\text{nm}$ . Such anion exchangers are described by Ugelstad, J., Mork, P.C., Berge, A., Ellingsen, T. and Kahn, A.A. in Emulsion Polymerization, Ed. by I. Piirma, pp.383-413, Academic Press, New York, 1983.

5 Preferably, the anion exchange resin is based on the material available under the trade mark Monobeads (Pharmacia Fine Chemicals), such as the Mono Q resins.

The use of strong anion exchangers  
10 characterised by particle size and porosity as broadly described above has been found to enable the removal of aggregates of gammaglobulin, anticomplementary activity, prekallikrein activator activity, Factor XII activity, plasmin and plasminogen activities, IgA and IgM from IgG  
15 containing starting materials such as Cohn Fraction II paste or powder. This process therefore contributes to the production of an intravenously tolerable IgG.

The use of microparticulate quaternary and  
20 tertiary strong anionic resins such as the Mono Q resins provides:

1. good mechanical stability,
2. resins which do not deform substantially with eluant conditions,
- 25 3. resins which show limited dispersity in terms of particle distribution and pore size distribution,
4. high loading capacities,
5. relative ease of regeneration due to high linear flow velocities, good mechanical stability etc.,
- 30 6. high resolution per unit time,
7. use of a very wide variety of eluant compositions, flow rate and temperature without compromising the mechanical and chemical stability of the resin and adversely prejudicing resolution and recovery,



8. increased recovery and decreased losses of biological activity due to the short residence and separation times.

5           Other resins with suitable properties for rapid large scale processing include silica based resins such as Accell QMA (Diosynth, Oss, Netherlands) and Spherosil QMA (Rhone Poulenc, France) and to a lesser extent the highly cross linked agarose supports such as  
10 Fast Flow Q (Pharmacia, Uppsala, Sweden).

          The present invention also provides a method employing dye affinity chromatography to remove kallikrein like esterase activity from IgG-containing  
15 materials. This method may be utilized alone, or in combination with the above-described anion exchange method.

          According to this aspect of the invention,  
20 there is provided a method for removal of kallikrein-like esterase and other protease enzyme or protease zymogen activity from an IgG-containing material, which comprises the steps of contacting the IgG-containing material with a protein-binding dye immobilised on a  
25 macroporous, mechanically stable gel support, and recovering the purified, kallikrein-depleted IgG-containing material.

          The invention further extends to purified IgG  
30 obtained by the above-described method.

          Preferably, the IgG-containing material is an IgG-rich material such as a Cohn Fraction II preparation (which optionally may have previously been subjected to  
35 the anion exchange step described above). The coupling

of the anion exchange step with the dye affinity chromatography procedure provides a method for the rapid separation of monomeric IgG from such IgG-rich preparations with good resolution to produce an IgG preparation suitable for intravenous injection. Preferably, the dye affinity chromatography is carried out before the anion exchange procedure.

The protein-binding dye is preferably a triazinyl-dye, typically of the Procion type (ICI Australia Operations) or its equivalent where produced by other manufacturers. Preferably also, the matrix used in the dye affinity chromatography is a semi-rigid matrix such as Fractogel (Merck, Darmstadt, Germany) or its equivalent such as TSK (Toyo, Soda), or Trisacryl (Reactifs I.B.F., France) or a similar material.

Dye affinity chromatography has been used in the purification of many enzymes and proteins. Generally chlorotriazine based dyes have been used. These have been covalently attached to a variety of supports including agarose, Sephadex, beaded cellulose, metal oxides, polyacrylamide, Sephacryl S200, Spheron, glass, microparticulate silica and agarose-acrylamide (Ultrigel) co-polymers (see Low, C. and Pearson, J., Methods in Enz. 104, Part C. 97-113). The chlorotriazine dyes have also been used in the purification or removal of some serum proteins in laboratory scale procedures.

The most commonly used dye-matrix has been Cibacron F3GA (Ciba Geigy) coupled to agarose. The Cibacron F3GA matrix has been used in the fractionation of different plasma proteins (Gianuzza, E. and Ainaud, P., Biochem J. (9182) 201 129-136), in the removal of

serum albumin and lipoproteins from other serum proteins (Travis, J., and Pannell, R., Clin.Chim.Acta. (1973) 49 49) in the purification of complement proteins (Gee, A. et al, J.Imm.Meths. (1979) 30 19) and in the  
 5 purifications of  $\alpha_2$  macroglobulin (Virca, G., et al., Anal.Bioch. (1978) 89 274). Blue dextran (Cibacron Blue F3GA coupled to dextran) has been used in the purification of serum lipoproteins, (Wille, L., Clin. Chim. Acta. (1976) 71 35) in the isolation of Factor X  
 10 (Viccian, L. and Tishkoff, G., Biochim.Biophys.Acta (1976) 434 199), and in the resolution of clotting factors II, VII, IX and X (Swart, A. and Hemker, H., Biochim.Biophys. Acta. (1970) 222 692). Procion Red (ICI Australia) coupled to agarose has been used to  
 15 extract plasminogen from serum. (Harris, N. and Byfield, P., FEBS Ltrs. (1979) 103 162).

In the method of the present invention, other triazine dyes such as those of the Procion series (ICI  
 20 Australia Operations), not previously utilized, have been coupled to mechanical stable gel matrices suitable for large-scale/industrial process-scale separations, such as Fractogel and Trisacryl and used to remove kallikrein-like activity from gammaglobulin solutions.  
 25 Fractogel consists of hydrophilic vinyl polymers and Trisacryl is a copolymer of an acrylic monomer and a bifunctional hydrophilic monomer. Advantages of these supports include:

- 30 i high mechanical stability,
- ii generally low non specific binding of protein to the gel matrix,

iii high binding capacity for dye. [Usually 1 ml of Fractogel or Trisacryl will bind 10 mg of a Procion dye from the MX series whereas 1 mL of agarose binds 2-4mg of dye (Lowe, C., and Pearson, J. Methods in Enz. 104 Part C 97-113).]

iv resistance to degradation by microorganisms.

Various Procion dyes linked to Fractogel have been shown to bind kallikrein like activity in gammaglobulin preparations; examples of dyes suitable for high affinity binding of kallikrein-like activity from IgG preparations are Navy HER, Navy HERD, Red HE 3B, Red MX5B, Red MX8B, Scarlet MX GR and Yellow MX GR.

Dye affinity chromatography as used in this invention offers several advantages over the use of immobilized substrates such as benzamidine for the removal of kallikrein activity. These include greater protein binding capacities, a low cost, general availability, ease of coupling to matrix materials, resistance to bacterial and enzymatic degradation and low toxicity. This makes dye affinity chromatography ideal for large scale protein purification.

Ethanol is a major component of Cohn Fraction II paste (25%). Consequently ethanol concentrations in the starting material can be significant. Concentrations of up to 10% ethanol have been shown to have little or no effect on resolution of components described above by both ion exchange and dye affinity chromatographies.

In a typical example of the ion exchange procedure of this invention, Mono Q resin is equilibrated in 20mM Tris Cl pH 8.0 (Buffer A), 60mM NaCl. The gammaglobulin solution prepared from Cohn Fraction II paste or powder is loaded onto the column and eluted from the column in Buffer A 60mM NaCl to produce a gammaglobulin solution free of aggregates, with a reduced content of dimeric gammaglobulin, low anticomplementary activity and no detectable IgA, IgM, PKA, Factor XII, plasmin or plasminogen activities. This is achieved with high recovery of gammaglobulin. This approach is illustrated by examples 1 and 3.

A typical procedure of dye affinity chromatography according to this invention employs the Procion dye such as Yellow MX GR coupled to Fractogel TSK-HW 55 (F) or TSK-HW 65(F). This dye affinity column is equilibrated in Buffer A, 60mM NaCl. The gammaglobulin solution prepared from Cohn Fraction II paste or powder is loaded onto the column and eluted from the column in Buffer A, 60mM NaCl, to produce a gammaglobulin solution with reduced kallikrein activity. Typically, a reduction of between 95 and 70% of kallikrein-like activity is seen with a high recovery of gammaglobulin. The residual activity can be removed by the ion exchange procedure previously described and is probably due to the presence of PKA. This approach is illustrated by Example 2.

A preparation of gammaglobulin free of aggregates (trimeric or above) anticomplementary activity, PKA, Factor XII, kallikrein, plasmin and plasminogen, IgA and IgM may be achieved in accordance with this invention by combining dye affinity and ion

exchange chromatography. In one preferred method, Yellow MX-GR-Fractogel and Mono Q resin is used. In this procedure the columns are equilibrated in Buffer A 60mM NaCl. The gammaglobulin solution is then passed  
5 through both columns and eluted with Buffer A 60mM NaCl to give gammaglobulin solution with the properties mentioned above. The preferred order of chromatography is dye affinity chromatography followed by ion exchange chromatography, since chromatography on the dye affinity  
10 support can generate low levels of aggregate IgG and consequently increase anticomplementary activity. This is removed by the ion exchange procedure. Furthermore, any dye that leaks from the affinity column is removed by the ion exchanger.

15 Use of other ion exchange resins (Accell QMA, Fast Flow Q) are illustrated by Examples 5, 6 and 7. Use of another dye (Red MX5B) which can be used is illustrated by Examples 4 and 6. In these Examples the  
20 dye has been coupled at a lower ligand density than in Example 2 and to a different matrix (Trisacryl GF 2000). The removal of kallikrein-like activity was less efficient in this Example whereas the recovery of IgG was greater.

25 EXAMPLE 1      Mono Q processing of Cohn Fraction II  
                         paste

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30 Chromatography was carried out on an FPLC system from Pharmacia which consisted of an LCC 500 microprocessor a UV1 control and optical unit, a REC 482 two channel chart recorder, 2 P500 pumps, a mixing chamber, an MV7 motorized valve and a 50ml superloop. All buffers were prepared using water that was quartz

distilled and deionized using a Milli Q system (Millipore, Bedford, M.O. USA). All buffers were degassed and filtered through a 0.45µm membrane. All buffer compounds were obtained from Sigma Chemicals Co. St. Louis, Miss., USA. The ion exchanger used was the Mono Q HR 16/10 from Pharmacia.

The Mono Q HR 16/10 (20 ml column volume) was connected to the FPLC system and equilibrated in 20mM Tris/Cl pH 8.0 (Buffer A) 60mM NaCl. 10 gram of Cohn Fraction II paste was taken up in 30 ml of Buffer A, 60mM NaCl and filtered through a 0.45µm membrane. The concentration of the filtered solution was 65 mg/ml. 17 ml of this solution was loaded onto the Mono Q HR 16/10 column at a flow rate of 3.0 ml/min. After loading the flow rate was increased slowly to 6 ml/min so that the back pressure did not exceed 3.5 MPa. Non retained protein was collected, analysed by HPSEC, and assayed for proteolytic and anticomplementary activities. Table 1 compares the properties of Cohn Fraction II solution before and after processing through the Mono Q.

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TABLE 1 Comparison of properties of Cohn FII solution  
with Mono Q processed Cohn FII

	COHN FII	Mono Q processed Cohn FII
% Aggregate }	1	0
% Dimer } 1	7	2
% Monomer }	92	98
Anticomplementary activity <sup>2</sup> (CH <sub>50</sub> /mg/hr)	11	<2
PKA (% B.O.B. ref. 2) }	13	0
Factor XII generated PKA } 3 (% B.O.B. ref. 2) }	4	0
Plasmin (ΔA405/min/mg) }	$3.4 \times 10^{-5}$	0
Plasminogen (ΔA405/min/mg) } 4	$9.5 \times 10^{-4}$	0
Kallikrein (ΔA405/min/mg) }	$7.7 \times 10^{-4}$	$5.6 \times 10^{-4}$

Recovery of IgG was 83%.

Footnotes to Table 1

<sup>1</sup> Aggregate levels of IgG in gammaglobulin solutions were measured by High Performance Size Exclusion Chromatography (HPSEC) on a 600 x 7.5mm TSK G3000SW column (Toya Soda Manufacturing Co., Japan). All chromatographic data were collected using one Model M6000A solvent delivery pump, a U6K universal injector, a Model M450 variable wavelength detector and a Model 730 Data Module all from Waters Assoc. Chromatography was carried out in 0.1 M Na phosphate pH 7.0 at a flow rate of 1 ml/min.

<sup>2</sup> Anticomplementary activity was measured by a microtitre plate immune haemolysis method based on



that in Weir (1978) (Handbook of Experimental Immunology Vol. 1 Sections 5A3-13, 3rd Ed. 1978 Blackwell). A pool of human AB sera, free of anti A<sub>1</sub> antibody served as source of complement and haemolysin and a 1.4% v/v suspension of sheep red blood cells (group ii) was used as the haemolysis indicator system. The IgG was incubated with 4 CH<sub>50</sub>-units of complement for one hour. The results are reported as complement units "consumed" (i.e. no longer available for the haemolysis reaction), in a one hour incubation with IgG, per mg IgG present.

PKA. The assay procedure is based on that of Imanari et al 1974 (Fogerty Int. Center Proc. No. 27 pp 205-213). The assay depends on the ability of prekallikrein activator (PKA) to convert prekallikrein (extracted from human plasma) to kallikrein, and on the monitoring of kallikrein activity by the generation of <sup>3</sup>H methanol from the synthetic substrate <sup>3</sup>H TAME (tosyl arginine methyl ester). The results are expressed in terms of a Bureau of Biologics Reference preparation for a 60 mg IgG/ml solution.

PKA generated from Factor XII breakdown was determined by first incubating the IgG samples in the presence of prekallikrein and dextran sulphate (based on the method of Tankersley, D.L. et al., Blood (1983) 62 448) and determining dextran sulphate-dependent PKA using the method described above.

4 Plasmin and kallikrein activities were measured  
spectrophotometrically ( $\Delta A_{405}$ , i.e. para  
nitroaniline generation) using the chromogenic  
substrates S2251 and S2303 respectively (Kabi  
5 Vitrum, Stockholm) and a Cary Model 15  
spectrophotometer.

All assays were carried out at 37°C in 50 mM Tris  
Cl, 50mM NaCl pH 8.0 (Buffer C) with a reaction  
10 volume of 200  $\mu$ l containing 40  $\mu$ l of substrate (1mM)  
and 40  $\mu$ l of sample. Reactions were stopped by the  
addition of 800  $\mu$ l of 2% acetic acid. Plasminogen  
activity was determined by pre-incubating the  
samples in the presence of 250 units per ml of  
15 streptokinase for 30 minutes at 37°C then  
determining streptokinase dependent plasmin  
activity.

20 EXAMPLE 2      Yellow MX GR - Fractogel processing of  
Cohn Fraction II paste

Yellow MX GR, a dichlorotriazine dye (referred  
to hereafter as dye), obtained from ICI Australia was  
coupled to the Fractogel support as follows:-

25 10g damp weight gel was suspended in 30 ml of  
water to which 0.15g of dye dissolved in 10 ml of water  
was added. 4 ml of 5M NaCl was added and the mixture  
incubated at room temperature with shaking for 1 hour.  
30 0.125 ml of 5M NaOH was added and incubation was  
continued with shaking at 30°C for 2 hours. Following  
this coupling procedure, the dye-Fractogel support was  
washed with 2-5 volumes of 6M Urea, 0.5M NaOH then  
thoroughly washed with water.

A Yellow MX GR - Fractogel column of 7 ml volume was prepared in a HR 10/10 column from Pharmacia. It was connected to the FPLC system described in Example 1 and equilibrated in 20mM Tris/Cl pH 8.0 (Buffer A) 60mM NaCl. 2 grams of paste was taken up in 6 ml of Buffer A, 60mM NaCl and filtered through a 0.45  $\mu$ m membrane. The concentration of the filtered solution was 50 mg/ml. 3.5 ml of this solution was loaded onto the dye column at a flow rate of 1.0 ml/min. The flow rate was increased to 2.0 ml/min after loading so that the back pressure did not exceed 0.7 MPa (100 psi). Non retained protein was collected. The non-retained protein solution contained 87% of the total protein loaded. This solution was analysed by HPSEC and assayed for anticomplementary and proteolytic enzyme activities. Table 2 compares the properties of Cohn Fraction II solution with dye column processed Cohn Fraction II.

**TABLE 2** Comparison of Cohn FII solution with Yellow  
MX-GR - Fractogel processed Cohn FII

	Cohn FII	Dye Processed Cohn FII
% Aggregate	1	1
% Dimer	7	10
% Monomer	92	89
ACA (CH <sub>50</sub> /min/mg)	11	19
PKA (% B.O.B. ref. 2)	17	7
Factor XII (% B.O.B. ref. 2)	5	3
- Dextran sulphate dependent PKA		
Plasmin ( $\Delta$ A405/min/mg)	0	0
Plasminogen ( $\Delta$ A405/min/mg)	$1.73 \times 10^{-3}$	$1.91 \times 10^{-4}$
Kallikrein ( $\Delta$ A405/min/mg)	$7.93 \times 10^{-4}$	$2.45 \times 10^{-4}$

Recovery of IgG was 87%.

For explanation of parameters see footnotes to Table 1.

**EXAMPLE 3** Mono Q, Yellow MXGR - Fractogel  
processing of Cohn Fraction II paste

The Mono Q HR 16/10, the Yellow MXGR Fractogel columns (7 ml of dye - Fractogel, packed in a Pharmacia HR 10/10 column) were connected in series so that the sample was loaded onto the Mono Q column and non  
5 retained protein passed directly onto the dye-Fractogel column. The columns were equilibrated in 20mM Tris/cl pH 8.0, (Buffer A), 60mM NaCl. 10 grams of Cohn FII paste was taken up in 30 ml of Buffer A, 60mM NaCl and  
10 filtered through a 0.45 $\mu$ m membrane. The concentration of the filtered solution was 65 mg/ml. 12 ml of this

solution was loaded onto the column. The initial flow rate was 2 ml/min but was decreased to 0.5 ml/min during loading to keep back pressure below 3.5 MPa. The flow rate was increased back to 2 ml/min as the back pressure dropped after loading. Non retained protein was collected. The non-retained protein solution contained 70% of the total protein loaded. This solution was analysed by HPSEC and assayed for anticomplementary and proteolytic enzyme activities. Table 3 compares properties of Cohn Fraction II solution with Mono Q-dye processed Cohn FII.

**TABLE 3** Comparison of Cohn FII solution with Mono Q-dye processed Cohn FII

	Cohn FII	Mono Q-Dye processed Cohn FII
% Aggregate	1	0
% Dimer	7	2
% Monomer	92	98
ACA (CH <sub>50</sub> /min/mg)	11	< 2
PKA (% B.O.B. ref 2)	3	0
Factor XII (% B.O.B. ref 2)	4	0
- Dextran sulphate dependent PKA		
Plasmin ( $\Delta$ A405/min/mg)	$3.4 \times 10^{-5}$	0
Plasminogen ( $\Delta$ A405/min/mg)	$9.5 \times 10^{-4}$	0
Kallikrein ( $\Delta$ A405/min/mg)	$7.7 \times 10^{-4}$	0

Recovery of IgG was 70%.

For explanation of parameters see footnotes to Table 1.

In the following Examples, the starting material was prepared by mixing Cohn Fraction II paste with 20mM Tris/Cl<sup>-</sup>, 60mM NaCl, pH 8.0 buffer at a ratio of 1:4 to give a final protein concentration of 54mg/mL. All resins were packed into 10ml columns and pre-equilibrated in 20mM Tris Cl<sup>-</sup>, 60mM NaCl pH 8.0.

EXAMPLE 4      Red MX5B - Trisacryl processing of Cohn Fraction II solution.

Red MX5B was coupled to Trisacryl GF 2000 by the method described in Example 2 except that a ratio of 10g damp gel to 0.05g of dye was used in this Example.

10ml of Cohn Fraction II solution was loaded onto the column at a rate of 1ml/min. The non-retained peak was collected and contained 95% of the total protein loaded. Some of the properties of the starting and Red MX5B Trisacryl-processed materials are compared in Table 4.

EXAMPLE 5      Accell QMA processing of Cohn F II solution.

4.3ml of Cohn FII solution was loaded onto a Accell QMA column at a flow rate of 2ml/min. The non-retained peak was collected and contained 95% of the total protein loaded. Some of the properties of the starting and Accell QMA-processed materials are compared in Table 4.

EXAMPLE 6      Combined Red MX5B - Trisacryl, Accell QMA processing.

10ml of the non-retained peak from Example 4 was loaded onto a Accell QMA column at 2ml/min. Recovery from the combined steps was calculated to be



TABLE 4 Summary of Data from Examples 4 to 7

Analytical Procedures	Cohn Fraction II Solution	Purification Process			
		Red MX5B- Trisacryl	Accell QMA	Red MX5B- Trisacryl and Accell QMA	Fast Flow Q
Protein Recovery (%)	100	95	95	87	84
HPSEC Analysis (%)					
Aggregate	0.5	1.4	0.1	0	0
Dimer	15.5	16.9	14.4	15.1	4.4
Monomer	84.0	81.7	85.5	84.8	95.6
Anticomplementary Activity (CH <sub>50</sub> /mg/hr)	7	11	1	2	3
* P.K.A. (% B.O.B. Ref.2)	6	3	0	0	0
* Factor XII (% B.O.B. Ref.2)	1	1	0	0	0
Plasmin Δ405/min/mg x 10 <sup>-4</sup>	0	0	0	0	0
Plasminogen Δ405/min/mg x 10 <sup>-4</sup>	3.15	0.8	0.8	0.34	0
Kallikrein Δ405/min/mg x 10 <sup>-4</sup>	3.37	2.12	2.12	0.76	2.10

\* Standardised for a 60mg/mL solution.

Refer to footnotes Table 1 for further description of analytical procedures.



EXAMPLE 8

1kg of Cohn Fraction II paste was dissolved in 1.5L of 20mM Tris/Cl<sup>-</sup>, 60mM NaCl pH 8.0 buffer and filtered. The filtrate was processed through a 300 mL Yellow MX GR-Fractogel column and a HR 16/10 Mono Q column in several batches. Column eluants were pooled and acidified, and ethanol was removed and the protein concentration adjusted to 6% w/v by diafiltration. The solution was subsequently sterile filtered and analysed for IgG subclass and IgA contents.

As will be seen from Table 5, all classes of IgG were present in the preparation in acceptable concentrations. Furthermore, the solution contained amounts of IgA below the level of detection of two of the three different commercial test kits for IgA (Table 6). However, the reasons for the clearly discrepant values between the three different kits, which were seen in duplicate assays, is not known at this stage.

TABLE 5

IgG Subclass Content of Dye-Monobead-Processed IgG, compared with Commercially Available IgG i.v. Preparations.

Purification Process	Concentration of IgG Subclasses (g/L) #			
	IgG <sub>1</sub>	IgG <sub>2</sub>	IgG <sub>3</sub>	IgG <sub>4</sub>
Yellow MXGR-Fractogel/Mono Q *	>23.5	>15.6	>1.88	0.24
<u>Conventional Process</u>				
<u>Manufacturer 1</u>				
Batch A	>23.5	>15.6	0.76	0.37
B	( 15.0	>15.6	>1.88	0.76
	(>23.5	>15.6	1.88	0.76
<u>Manufacturer 2</u>	>23.5	>15.6	>1.88	0.24
<u>Manufacturer 3</u>	>23.5	>15.6	>1.88	0.09

# In 60g IgG/L IgG solution, assayed by Miles ACCRA subclassing kits.

\* As described in Example 8.

Table 6

IgA Content of Dye-Monobead-Processed IgG Compared with Commercial IgG i.v. Preparations.

Purification Process	IgA Concentration (µg/mL)		
	Test Kit		
	Behring IgA Nor Partigen	ICL (Boehringer Mannheim) Multitest	Miles ACCRA
Yellow MXGR-Fractogel/Mono Q *	<60	<210	240
<u>Conventional Process</u>			
Batch 1	220	510	150
2	270	410	250

\* As described in Example 8.

CLAIMS:

1. A method for the purification of an IgG containing material, which comprises the steps of:
  - (i) fractionating an immunoglobulin G-containing material on a microparticulate, strong anion exchange resin having meso- and macro- porous surfaces, and
  - (ii) recovering a purified IgG fraction from said resin by elution.
2. A method according to claim 1, wherein said resin is a microparticulate quaternary or tertiary strong anion exchange resin.
3. A method according to claim 1 or claim 2, wherein said resin has nominal particle diameters of 3, 5, 10, 30 and 90  $\mu\text{m}$ , with narrow particle diameter distribution and narrow pore size distribution, based on porosity of  $> 10\text{nm}$ .
4. A method according to any one of claims 1 to 3, wherein said immunoglobulin G-containing material is Cohn Fraction II paste or powder.
5. A method according to any one of claims 1 to 4 wherein said anion exchange resin is Mono Q resin.
6. A purified IgG fraction prepared by the method of any one of claims 1 to 5.
7. A method for removal of Kallikrein-like esterase and other protease enzyme or protease zymogen activity from an IgG-containing material which comprises the steps of:

- (i) contacting the IgG-containing material with a protein-binding dye immobilised on a macroporous, mechanically stable gel support, and
- (ii) recovering the enzyme and zymogen-depleted IgG-containing material.

8. A method according to claim 7, wherein said IgG-containing material is a purified IgG fraction prepared by the method of any one of claims 1 to 5.

9. A method according to claim 7 wherein said IgG-containing material is Cohn Fraction II paste or powder.

10. A method according to claim 7, wherein said enzyme and zymogen-depleted IgG-containing material is further purified by the method of any one of claims 1 to 5.

11. A method according to any one of claims 7 to 10, wherein said protein-binding dye is a triazinyl protein-binding dye.

12. A method according to claim 11, wherein said triazinyl-dye is a dye of the Procion type.

13. A method according to claim 12, wherein said dye is selected from the group consisting of Navy HER, Navy HERD, Red HE 3B, Red MX 58, Red MX 8B, Scarlet MX GR and Yellow MX GR.

14. A method according to any one of claims 7 to 13, wherein said gel support is a semi-rigid or rigid matrix.

15. A method according to claim 14, wherein said matrix is composed of Fractogel and its equivalent or Trisacryl material.

16. A kallikrein, plasmin and plasminogen-depleted IgG-containing material prepared by the method of any one of claims 7 to 15.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 86/00139

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> : special classification symbols apply indicate art <sup>1</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC Int. Cl <sup>4</sup> C07K 15/12, 3/18		
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>2</sup>		
Classification System	Classification Symbols	
IPC US Cl.	C07K 15/12, 3/18, C07G 7/00 260/112B	
Documentation Searched other than Minimum Documentation to the extent that such documents are included in the fields searched <sup>3</sup>		
AU: IPC as above		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>4</sup></b>		
Category <sup>5</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	US, A, 4434093 (ZOLTON <u>et al</u> ) 28 February 1984 (28.02.84)	(1-6)
X	US, A, 4100149 (MEILLER <u>et al</u> ) 11 July 1978 (11.07.78)	(1-6)
X	Separation News, Volume 1, published 1982, by Pharmacia Fine Chemicals AB (Uppsala), 'IgG, IgA and IgM: improved separation by use of the Pharmacia FPLC system'.	(1-6)
X	Journal of Chromatography, Volume 319, No 1, issued 1985 January (Amsterdam) P. Clezardin, <u>et al</u> "One-step Procedure for the Rapid Isolation of Mouse Monoclonal Antibodies and their Antigen Binding Fragments by Fast Protein Liquid Chromatography on a Mono Q Anion-Exchange Column", see pages 67-77	(1-6)
P,X	Journal of Chromatography, Volume 359, issued 1986 May (Amsterdam), B.Pavlu, <u>et al</u> , "Rapid Purification of Monoclonal Antibodies by High Performance Liquid Chromatography", see pages 449-460.	(1-6)
P,X	AU, A, 51030/85 (ORTHO DIAGNOSTIC SYSTEMS, INC.) : 19 June 1986 (19.06.86)	(1-6)
Continued		
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>14</sup> Special categories of cited documents</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"A" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
22 August 1986 ( 22.08.86)	28 August 1986 (28.08.86)	
International Searching Authority	Signature of Authorized Officer	
AUSTRALIAN PATENT OFFICE	J.H. CHAN	

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
P,X	Vox Sanguinis, Volume 49, issued 1985 (Basel), R.P. Zolton, <u>et al</u> , "Removal of Hepatitis B Virus Infectivity from Human Gamma-Globulin Prepared by Ion-Exchange Chromatography", see pages 381-389	(1-6)
A	US, A, 4451487 (VETTER <u>et al</u> ) 29 May 1984 (29.05.84)	(7-16)
A	Protides of the Biological Fluids, Volume 32, issued 1984 (published in 1985) by Pergamon Press, P. Arnaud, <u>et al</u> , "Combined Pseudo-Ligand Affinity Chromatography as a General Method for Plasma Protein Purification", see pages 1117-1120	(7-16)
P, A	US, A, 4546161 (HARVEY <u>et al</u> ) 8 October 1985 (08.10.85)	(7-16)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON  
INTERNATIONAL APPLICATION NO. PCT/AU 86/00139

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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Patent Document  
Cited in Search  
Report

Patent Family Members

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US 4546161                      WO 7900541

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	ES 451047	FR 2321932	GB 1513195
	IT 1066221	JP 52057200	NL 7609562
	NO 762959	NZ 181884	SE 7609479
	US 4100149	YU 2096/76	

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